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Treating ram sperm with cholesterol-loaded cyclodextrins improves cryosurvival☆

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ABSTRACT

Acceptable fertility using cryopreserved ram sperm is currently only achieved using laparoscopic intrauterine insemination. Improving the cryosurvival of ram sperm may permit greater fertility rates using more practical techniques. This study was conducted to determine if treating ram sperm with six different cyclodextrins pre-loaded with cholesterol (CLC), prior to cryopreservation increases sperm cryosurvival and if this technology can be used with neat semen. Subsequent experiments evaluated how adding CLC to sperm affected sperm cholesterol content, sperm osmotic tolerance limits, sperm post-thaw survival after incubation and the capacity of sperm to bind to zona pellucidae of cattle and sheep oocytes. Sperm treated with 2-hydroxypropyl-β-cyclodextrin prior to cryopreservation exhibited greater percentages of motile sperm (62%) compared to the control (no CLC treatment) samples (43%, $P < 0.05$), after thawing. In addition, samples treated with methyl-β-cyclodextrin exhibited percentages of motile and viable sperm similar to samples treated with 2-hydroxypropyl-β-cyclodextrin. Other CLC-treated samples were similar to the control. The CLC concentration that optimized sperm cryosurvival was 2 mg CLC/120 × 10^6 sperm for both methyl-β- and 2-hydroxypropyl-β-cyclodextrin when added to neat semen prior to cryopreservation. Addition of 2 mg CLC not only maintained greater percentages of motile sperm compared to the control samples, but maintained greater percentages of motile sperm during a 3 h incubation after thawing. In addition, 2-hydroxypropyl-β-cyclodextrin pre-loaded with cholesterol maintained greater percentages of viable sperm (33%), than control sperm (18%; $P < 0.05$). Treating ram sperm with CLC increased the sperm cholesterol content > 1.9-fold and although some cholesterol was lost from the sperm during cooling and cryopreservation, the cholesterol content remained greater in CLC-treated sperm after cooling and after thawing than in control sperm ($P < 0.05$). In addition, CLC-treated sperm maintained greater percentages of motile sperm through a wide range of osmotic solutions (150 and 425 mOsm) while control sperm lost motility in solutions outside a more narrow range (270 to 370 mOsm). Greater numbers of CLC-treated sperm bound to zona pellucida than control sperm ($P < 0.05$), although number of sperm binding cattle and sheep oocytes, was similar ($P > 0.05$). In conclusion, treating ram sperm with CLC increases sperm cryosurvival rates and sperm longevity after thawing. It also increases the cholesterol content, osmotic tolerance, and zona-binding capabilities of sperm. Finally, CLCs can be added to neat semen, making this technology feasible for practical application using current cryopreservation techniques for ram semen.

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1. Introduction

Artificial insemination is the main technology for rapidly dispersing valuable genetics of livestock. The method is simple, successful and economical for establishing genes in a population compared to embryo transfer or natural mating (Vishwanath, 2003). The ability to cryopreserve sperm plays an important role in artificial insemination of livestock and for conservation of endangered species, as cryopreserved sperm can be stored for long periods of time, and can be sent worldwide facilitating the exchange of genetic material between distant animal populations.

Although several cryopreservation protocols and freezing diluents have been developed to cryopreserve ram sperm (reviewed by Salamon and Maxwell, 2000), only about half of the sperm survive the process due to cryodamage and the surviving sperm exhibit reduced fertilizing capacity (Curry and Watson, 1994). Cell cryo-damage occurs due to intracellular ice formation (Mazur, 1984), membrane alterations induced by phase transitions that occur when membranes are cooled (Amann and Pickett, 1987; Hammerstedt et al., 1990; Watson, 2000; Medeiros et al., 2002), and osmotic stresses due to cryoprotectant addition and removal, as well as the cryopreservation process itself (Curry and Watson, 1994; Gilmore et al., 1995; Holt, 2000; Guthrie et al., 2002; Morris et al., 2007). Recent evidence indicates that due to the limited free water in a sperm, intracellular ice formation does not induce significant cryodamage (Morris, 2006; Morris et al., 2007). Therefore, focusing on methods to improve membrane function at low temperatures may be key to improving the cryosurvival of sperm.

As sperm are cooled the membrane phase transition occurs, lipids aggregate into micro-domains, which alter membrane function and induce membrane gaps between the gel and remaining fluid membrane domains (Amann, 1999). Cholesterol controls membrane structure by interacting with the phospholipid hydrocarbon chains (Darin-Bennett and White, 1977), and at temperatures below the phase transition, forces the chains apart, making the membrane more stable (Quinn, 1989).

Cyclodextrins are cyclic oligosaccharides which possess an external hydrophilic face and an internal hydrophobic core (Christian et al., 1997). These molecules have a high affinity for sterols *in vitro*, and if they are pre-loaded with cholesterol can insert cholesterol into cell membranes (Navratil et al., 2003). Several authors have reported increased cryosurvival rates when stallion (Combes et al., 2000; Moore et al., 2005), bull (Purdy and Graham, 2004), donkey (Alvarez et al., 2006), pig (Galantino-Homer et al., 2006), and ram (Morrier et al., 2004) sperm were treated with methyl-β-cyclodextrin pre-loaded with cholesterol, prior to cryopreservation.

In an effort to learn more about how added cholesterol affects sperm, studies were conducted to determine if treating ram sperm with different cyclodextrins pre-loaded with cholesterol (CLC), prior to cryopreservation, could increase sperm cryosurvival rates, and its influence on ram sperm quality and physiology. These studies were conducted in order to develop a cryopreservation technique to improve ram sperm cryosurvival and that could be used commercially with neat ram semen.

2. Materials and methods

2.1. Materials

All chemicals were reagent grade and purchased from Sigma–Aldrich, St. Louis, MO, USA; except for SYBR-14 and propidium iodide (PI) which were purchased from Invitrogen, Eugene, OR, USA.

2.2. Cyclodextrin preparation

Six different cyclodextrins (α-cyclodextrin, α-cyclodextrin hydrate, β-cyclodextrin, β-cyclodextrin hydrate, 2-hydroxypropyl-β-cyclodextrin or methyl-β-cyclodextrin) were pre-loaded with cholesterol as described by Purdy and Graham (2004). Briefly, a 0.45 mL aliquot of cholesterol dissolved in chloroform (200 mg cholesterol per 1 mL chloroform) was added to 2 mL of methanol containing 1 g cyclodextrin and each mixture stirred until the combined solution was clear. The solvents were removed, the resulting crystals allowed to dry for 24 h, and the crystals were stored at 22 °C until use. A working solution of each CLC was prepared by adding 50 mg of CLC to 1 mL of Tris diluent (Purdy and Graham, 2004) at 37 °C and mixing the solution using a vortex mixer for 30 s. For the negative control, methyl-β-cyclodextrin (not pre-loaded with cholesterol) was prepared similarly, but no cholesterol solution was added to the cyclodextrin solution.

2.3. Semen collection

Semen was collected by electro-ejaculation September through March, as described by Evans and Maxwell (1987), from adult rams of Southdown and Rambouillet breeds housed at Colorado State University, Fort Collins, CO, USA. Rams were fed a diet providing 100% of their nutritional needs, and provided water *ad libitum*. All animal care and semen collection procedures were approved by the Animal Care and Use Committee of Colorado State University.

After collection, semen was diluted 1:1 with Tris diluent (300 mM tris[hydroxymethyl]aminomethane, 95 mM citric acid monohydrate, 28 mM D-(+)-Glucose, pH 7.0) and transported to the laboratory in an insulated Styrofoam box and used within 45 min of collection.

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2.4. Analysis of spermatozoa

The percentages of motile and progressively motile sperm in each sample were determined using a CASA system (Hamilton Thorne Research, IVOS version 10.7 s, Bedford, MA, USA) with settings of 30 frames acquired to avoid sperm track overlapping, minimum contrast 80, minimum velocity of average path = 14 μm/s, straightness = 40%, non-motile head size 3, non-motile head intensity 96. For each sample, 6 μL sub-samples were placed on slides and a minimum of 200 sperm per sub-sample analyzed.

The percentage of viable (plasma membrane intact; PMI) sperm in each sample was determined using flow cytometry, as described by Purdy and Graham (2004). Briefly, sperm were stained for flow cytometric analysis by transferring a 0.1 mL aliquot from each sample into a tube containing 0.45 mL Tris-BSA (6 mg BSA/mL) diluent, 8.5 μL SYBR-14 (10 μM solution in DMSO) and 5 μL PI (1.5 mM solution in distilled water). The samples were incubated for 10 min at 22 °C and filtered through a 40 μm nylon mesh before being analyzed using an Epics V flow cytometer (Beckman Coulter, Inc., Fullterton, CA, USA) equipped with an argon laser tuned to 488 nm at 100 mW power. Fluorescence from 50,000 sperm was measured using a 515 long pass filter, a 525 nm band pass filter to detect SYBR-14, a 590 nm dichroic mirror and a 630 nm long pass filter to detect PI. Using this protocol, all sperm stain with SYBR-14, permitting cells to be distinguished from egg yolk particles, but only non-viable sperm stain with PI.

2.5. Experiment 1. Determining the optimal cyclodextrin for ram sperm cryopreservation

Ejaculates from nine rams were used in this study. Immediately upon reaching the laboratory, the concentration of spermatozoa in each ejaculate was determined photometrically (Foote, 1972) and each sample was diluted to 120×10^6 sperm/mL with the Tris diluent. Each sperm sample was then split into eight treatment aliquots (0.5 mL each). One of the aliquots served as a control (no-CLC treatment) and one was treated with methyl-β-cyclodextrin (not cholesterol–loaded). The other aliquots were treated with α-cyclodextrin, α-cyclodextrin hydrate, β-cyclodextrin, β-cyclodextrin hydrate, 2-hydroxypropyl-β-cyclodextrin, or methyl-β-cyclodextrin each of which were pre-loaded with cholesterol. All treatments received 2 mg cyclodextrin/120×10^6 cells. Samples were incubated for 15 min at 22 °C, after which each sample was diluted to 60×10^6 sperm/mL, with Tris diluent. The samples were then diluted 1:1 (v/v) with Tris diluent containing 30% egg yolk and 10% glycerol (resulting in final concentrations of egg yolk = 15%, glycerol = 5% and sperm = 30×10^6 cells/mL).

The sperm were then cooled to 5 °C over 2 h, packaged into 0.5 mL straws and frozen in liquid nitrogen vapor, with the straws horizontally suspended 4.5 cm above the liquid nitrogen for 13 min, before being plunged into liquid nitrogen for storage. The estimated initial cooling rate for the straws was −50 °C/min.

Two straws from each treatment were thawed in a water bath at 37 °C for 30 s, prior to analysis. The contents of one straw were used to determine the percentage of motile sperm in each treatment, while those of the second straw were used to determine the percentage of sperm possessing an intact plasma membrane.

2.6. Experiment 2. Determination of the optimal CLC concentration

Two separate experiments were conducted in order to determine the CLC concentration which optimized ram sperm cryosurvival. In the first experiment, ejaculates from 10 rams were collected, brought to the laboratory, diluted to 120×10^6 cells/mL with Tris diluent and split into five treatment aliquots of 0.5 mL each. A total of 0, 0.5, 1, 2, and 4 mg methyl-β-cyclodextrin preloaded with cholesterol was added per 120×10^6 cells and the samples incubated for 15 min. After incubation each sample was diluted to 60×10^6 cells/mL in Tris diluent and then diluted 1:1 with Tris-egg yolk extender (resulting in a final concentration of egg yolk of 15% and glycerol of 5%), frozen, thawed and evaluated as described above.

In the second experiment, sperm were treated with two amounts (chosen from the previous experiment) of the two cyclodextrins which resulted in the best cryosurvival from experiment 1. Eleven ejaculates were collected as described and the sperm diluted to 120×10^6 sperm/mL with the Tris diluent. Samples were split into five treatment aliquots of 0.5 mL each. One aliquot was used as the control (no CLC treatment), and each one of the other four aliquots was treated with either 2-hydroxypropyl-β-cyclodextrin or methyl-β-cyclodextrin, each at either 1 or 2 mg CLC/120×10^6 sperm. Samples were incubated for 15 min at 22 °C, after which each sample was diluted to 60×10^6 cells/mL in Tris diluent, diluted 1:1 with Tris-egg yolk extender, frozen, thawed and evaluated as described above.

2.7. Experiment 3. Addition of CLCs to raw ejaculates

Immediately after collection, the concentration of sperm in each of 20 ejaculates was determined and each ejaculate split into three aliquots, one aliquot was used as a control (no CLC treatment) and the other two were treated with either 2-hydroxypropyl-β-cyclodextrin or methyl-β-cyclodextrin (each preloaded with cholesterol) at 2 mg CLC/120×10^6 sperm, and incubated for 15 min at 22 °C. After incubation, 60×10^6 cells were added to 1 mL of the Tris extender and this was diluted 1:1 with the Tris-egg yolk extender and the sperm were frozen, thawed and evaluated as described above.

2.8. Experiment 4. Resistance of sperm to incubation (sperm thermoresistance)

The longevity of sperm motility for control (non-CLC treated) and CLC-treated ram sperm was determined for cryopreserved sperm incubated after thawing for up to 3 h. Seven ejaculates were collected and cryopreserved at a concentration of 200×10^6 sperm/mL, as described previously. After thawing, six 50 μL aliquots, from each ejaculate, were diluted to 30×10^6 sperm/mL with Tris-BSA, and the samples incubated at 38.5 °C, in an atmosphere of 5% CO_2 in air

and 100% humidity for up to 3 h. One aliquot was removed and at 0, 30, 60, 90, 120 and 180 min, and the motility of the sample determined using CASA, as described previously.

2.9. Experiment 5. Cholesterol content of CLC-treated ram sperm

The cholesterol content of fresh, cooled and frozen-thawed ram sperm was determined as described by Moore et al. (2005). Briefly, four separate ejaculates were collected, diluted with a Tris diluent, the sperm concentration determined, split into three aliquots, and treated with no CLC (control), 2-hydroxypropyl-β-cyclodextrin, or methyl-β-cyclodextrin (each preloaded with cholesterol), as described above. After incubation, 400×10^6 sperm were taken from each aliquot, to determine the cholesterol content of the fresh sperm, and the remainder of each aliquot cooled to 5 °C, as described above. Upon reaching 5 °C, another 400×10^6 sperm were removed from each sample to determine the cholesterol content of cooled sperm, and the remainder of each aliquot cryopreserved, as described above. After thawing, 400×10^6 sperm were removed from each sample to determine the cholesterol content of frozen-thawed sperm

The fresh (after incubation with CLC), cooled and cryopreserved sperm were centrifuged through 45% Percoll at $975 \times g$ for 20 min, the supernatant discarded. The sperm pellet re-suspended in 5 mL of phosphate buffered saline (PBS; 171 mM NaCl, 3 mM KCl, 10 mM Na_2HPO_4, and 2 mM KH_2PO_4, pH 7, 300 mOsm) and the samples centrifuged a second time, as described above, and the supernatant discarded. The sperm pellet was suspended in 0.3 mL of PBS, the sperm concentration was determined by spectrophotometry, and the samples stored at −20 °C until analysis.

The amount of cholesterol in each sample was determined using the cholesterol liquicolor enzymatic assay (Stanbio, Boerne, TX, USA) as described by Navratil et al. (2003). Briefly, samples were diluted 1:1 (v:v) with lysate buffer (0.4% Triton X-100 in PBS) for 1 h at 22 °C to solubilize the plasma membranes. Samples were then diluted 1:5 (v:v) with reagent and incubated for 25 min at 37 °C. Each sample was then placed into a 1.5 mL eppendorf tube and centrifuged for 3 min at 14,000 rpm, using an Eppendorf centrifuge, Model 5415C (Brinkmann Instruments, Inc., Westbury, NY, USA) to remove sperm debris (Moore et al., 2005). The supernatant was collected and analysed for cholesterol content via spectrophotometer (Spectronic Genesys 5, Phoenix, AZ, USA) at 500 nm. The amount of cholesterol in each sample was determined from a standard curve.

2.10. Experiment 6. Effect of CLC treatment on the osmotic tolerance of ram sperm

This experiment determined the ability of control and CLC-treated ram sperm to maintain motility after exposure to anisosmotic Tris-BSA (6 mg/mL) solutions and then being returned to isosmotic conditions, to evaluate the osmotic tolerance of the sperm. Eight different ejaculates were collected, diluted (dilution 1:1; v:v) and the sperm concentration determined, as described above. If the sperm concentration was lower than 10^9 sperm/mL, the samples were centrifuged (7 min 30 s, $275 \times g$) to increase the sperm concentration.

The ejaculates were split into five fractions: control (non CLC-treated), and treated with either 2 or 4 mg/120×10^6 sperm of each of two different cyclodextrins (2-hydroxypropyl-β-cyclodextrin and methyl-β-cyclodextrin) pre-loaded with cholesterol. Control samples were diluted with Tris diluent (pH 7.0; 300 mOsm) to a concentration of 10^9 sperm/mL. CLC-treated samples were incubated with CLCs at 22 °C for 15 min, and then diluted to 1×10^9 sperm/mL. The osmotic tolerance of the sperm was then determined by diluting sperm into Tris diluents having different osmolality (50, 75, 150, 225, 270, 300, 350, 370, 425, 600, 1200 and 2400 mOsm), as described by Guthrie et al. (2002), with slight modifications. Briefly, a 20 μL aliquot of each sperm sample was added to 150 μL of one of the 13 anisosmotic solutions, yielding a final sperm concentration of approximately $100–120 \times 10^6$ sperm/mL. Sperm incubated in the anisosmotic solution for 5 min at 22 °C, and then were returned to near isosmolality (261–323 mOsm) by transferring a 100 μL aliquot from each anisosmotic sample into volumes of Tris solutions (supplemented with 1.2 mg of BSA/mL) to restore sperm to an isosmotic environment (Fig. 1). Sperm motility was then evaluated, as described previously.

2.11. Experiment 7. CLC-treatment and ability of ram sperm to bind the zona pellucida

The ability of cryopreserved ram sperm to bind to the zona pellucida of oocytes of sheep and cattle was conducted as described by Moore et al. (2005), with some modifications.

2.11.1. Sperm preparation

Ejaculates from seven rams were collected, treated with CLC (control or treated with either 2-hydroxypropyl-β-cyclodextrin or methyl-β-cyclodextrin, each preloaded with cholesterol) and cryopreserved at a concentration of 200×10^6 sperm/mL, as described previously.

For each ram, a single straw from each treatment (control, or treated with methyl-β-cyclodextrin or 2-hydroxypropyl-β-cyclodextrin each pre-loaded with cholesterol) was thawed, the contents diluted 1:3 (v:v) with a modified Tyrode's medium (TALP; Graham et al., 1986), washed by centrifugation at $365 \times g$ for 5 min, and the cells suspended in 1 mL of TALP containing 25 μg/mL Hoechst 33342. The sperm incubated with the stain for 15 min at 37° C, after which the sperm were washed again ($365 \times g$ for 5 min) and suspended to a final concentration of 2×10^6 sperm/mL in TALP. Five microliter aliquots (10,000 sperm) from each sperm suspension were added to each of two droplets containing five oocytes each.

2.11.2. Oocyte preparation

Immature cattle and sheep oocytes were recovered from ovaries obtained from a local abattoir and transported to the laboratory in saline at 37 °C within 4–5 h of collection. Follicles ranging from 2 to 6 mm were aspirated using an

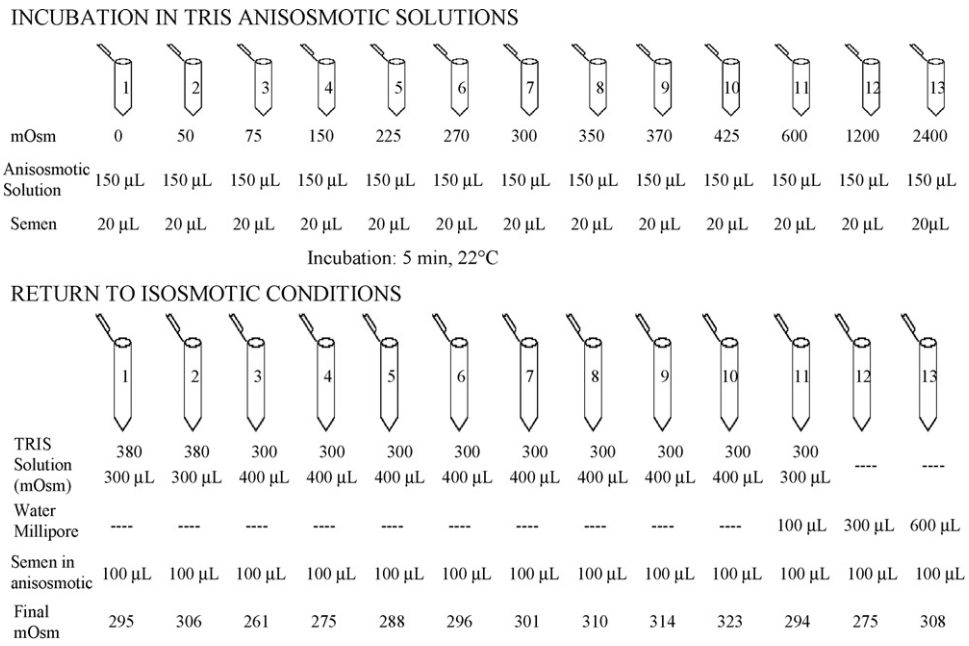

Fig. 1. Protocol for incubating untreated ram sperm and ram sperm treated with different amounts of two cholesterol-loaded cyclodextrins (CLCs) in anisosmotic conditions and then returning the sperm to near isosmotic, prior to assessing the percentage of motile sperm in each sample.

18 g needle and the aspirate observed using a Bausch and Lomb, StereoZoom 6, stereomicroscope (Cambridge Instruments, Buffalo, NY, USA). Observed oocytes were placed into TALP after which the cumulus cells were removed using a vortex mixer at maximum speed for 2 min. This process was repeated as many times as needed until oocytes were completely denuded of cumulus cells. The oocytes were washed in TALP and stored at 5 °C in a hyperosmotic salt solution (1.5 M $MgCl_2 \times 6{\cdot}H_2O$; 40 mM Hepes; 0.1% PVP; pH 7.4; Yanagimachi et al., 1979) until use. Bovine oocytes (two drops with five oocytes each/sperm treatment) were inseminated by sperm from all seven ram ejaculates. Ovine oocytes were used with sperm from only ram five ejaculates, and for one, only a single drop (with five oocytes) was inseminated.

2.11.3. Zona binding assay

Prior to adding the sperm, the oocytes were washed several times in TALP and then five oocytes randomly placed into 45 µL droplets of TALP and the oocytes incubated for 1 h at 38.5 °C in an atmosphere of 5% CO_2 in air, and 100% humidity, while the sperm were thawed and prepared. Sperm were added to the oocytes and the gametes incubated for 2 h. The oocytes were then washed vigorously in six droplets of TALP using a small-bore fire polished glass pipette to remove loosely bound sperm. Oocytes from the same droplet were placed onto glass slides and covered with a cover glass supported by a mix of paraffin wax and petroleum jelly. Oocytes were viewed using an epifluorescent microscope (Eclipse E800, Nikon Instruments, Melville, NY) equipped with a 360/40 nm band pass excitation filter and a 460/50 nm band pass emission filter. The total number of sperm bound to each zona pellucida was determined at 400x magnification.

2.12. Statistical analyses

Percentage data (percent motile and plasma membrane intact sperm) were transformed using Arcsine prior to analysis. Treatment differences, between cyclodextrin

Table 1
Percentages of total motile, progressively motile and plasma membrane intact (PMI) cells after cryopreservation of ram spermatozoa pre-treated with six different cholesterol loaded cyclodextrins (CLC), each at 2 mg CLC/120 × 10^6 sperm.

Treatment	Total motile sperm (%)	Progressively motile sperm (%)	PMI (%)
Control	43^{b,c}	32^{b,c}	27
Cyclodextrin non-cholesterol loaded	31^{c}	23^{c}	25
α-Cyclodextrin	47^{b,c}	36^{b,c}	32
α-Cyclodextrin hydrate	40^{b,c}	33^{b,c}	25
β-Cyclodextrin	44^{b,c}	34^{b,c}	30
β-Cyclodextrin hydrate	40^{b,c}	32^{b,c}	23
2-Hydroxypropyl- β-cyclodextrin	62^{a}	50^{a}	43
Methyl-β-cyclodextrin	54^{a,b}	42^{a,b}	39
SEM	8	7	6

^{a,b,c}Means with different superscripts within a column differ, $P < 0.05$.

Table 2
Percentages of total motile and plasma membrane intact (PMI) cells after cryopreservation when ram spermatozoa were treated with different concentrations of methyl-β-cyclodextrin pre-loaded with cholesterol (CLC).

CLC concentration (mg/120 × 10^6 sperm)	Total motile sperm (%)	PMI (%)
0	28[b]	24[b]
0.5	40[a]	35[a,b]
1	46[a]	45[a]
2	45[a]	47[a]
4	45[a]	36[a,b]
SEM	8	5

[a,b]Means with different superscripts within a column differ, $P < 0.05$.

Table 4
Percentages of total motile, progressively motile and plasma membrane intact (PMI) cells when raw ram ejaculates were treated with 2 mg of methyl-β-cyclodextrin or 2-hydroxypropyl-β-cyclodextrin/120 × 10^6 sperm, each pre-loaded with cholesterol (CLC) and then cryopreserved.

Treatment	Total motile sperm (%)	Progressively motile sperm (%)	PMI (%)
Control	32[b]	25[b]	18[b]
Methyl- β-cyclodextrin	42[a]	34[a]	22[b]
2-Hydroxypropyl- β-cyclodextrin	50[a]	39[a]	33[a]
SEM	5	4	4

[a,b]Means with different superscripts within a column differ, $P < 0.05$.

treatment and between CLC concentrations (in experiments evaluating multiple levels) were determined by analysis of variance (Statistical Analysis and Systems, 1985), and treatment means were separated using Student–Newman-Kuels (SNK) multiple range test. Tables present the non-transformed data.

In experiment 4, CLC treatment differences in the percentage of total motile sperm within each time period (0, 30, 60, 90, 120 and 180 min) and within each CLC treatment across time were determined by analysis of variance and treatment means were separated using SNK (Statistical Analysis and Systems, 1985).

In experiment 6, motility data for each ejaculate were normalized to the percentage of motile sperm in the control sample (300 mOsm), of that ejaculate, prior to analysis by ANOVA (Statistical Analysis and Systems, 1985), and individual treatment means were separated using SNK. Treatments were considered to be different if $P < 0.05$.

3. Results

3.1. Experiment 1. Determining the optimal cyclodextrin for ram sperm cryopreservation

Sperm treated with 2 mg of the 2-hydroxypropyl-β-cyclodextrin pre-loaded with cholesterol, exhibited greater ($P < 0.05$) percentages of total motile (+19%) and progressively motile (+18%) cells, compared to control sperm (Table 1). Similar trends were observed in the percentages of PMI sperm after cryopreservation, with 2-hydroxypropyl-β-cyclodextrin treated samples exhibiting greater percentages of PMI (+16%) sperm than control samples (Table 1). Sperm treated with non-cholesterol loaded cyclodextrin, or with cholesterol-loaded β-cyclodextrin, β-cyclodextrin hydrate, α-cyclodextrin and α-cyclodextrin hydrate, resulted in percentages of total motile sperm, progressive motile and PMI sperm that were similar to control sperm ($P > 0.05$; Table 1). When the control sample was compared to only the methyl-β- and 2-hydroxypropyl-β-cyclodextrin treatments (the cyclodextrins providing the greatest cryosurvival rates), sperm treated with either CLC resulted in greater post-thaw percentages of motile, progressively motile and PMI sperm, than control sperm ($P < 0.05$).

3.2. Experiment 2. Determination of the optimal CLC concentration

All CLC concentrations tested produced greater percentages of total motile sperm ($P < 0.05$) when sperm were treated with methyl-β-cyclodextrin than control sperm (Table 2). However, while 1 or 2 mg CLCs/120 × 10^6 sperm resulted in greater percentages of PMI sperm after cryopreservation ($P < 0.05$), samples treated with 0.5 or 4 mg CLCs/120 × 10^6 sperm did not ($P < 0.05$).

Sperm treated with either methyl-β-cyclodextrin or 2-hydroxypropyl-β-cyclodextrin (1 or 2 mg CLC/120 × 10^6 sperm), exhibited greater percentages of total motile, progressive motile and PMI sperm compared to control sperm ($P < 0.05$; Table 3).

3.3. Experiment 3. Addition of CLCs to raw ejaculates

When CLCs were added to raw ejaculates (2 mg of methyl-β- or 2-hydroxypropyl-β-cyclodextrin loaded with cholesterol/120 × 10^6 sperm), greater percentages of total motile sperm and progressively motile sperm were achieved after thawing, compared to control sperm

Table 3
Percentages of total motile, progressively motile and plasma membrane intact (PMI) cells when ram spermatozoa were treated with 0, 1 or 2 mg of methyl-β-cyclodextrin or 2-hydroxypropyl-β-cyclodextrin, each pre-loaded with cholesterol (CLC)/120 × 10^6 sperm, and then cryopreserved.

Treatment	CLC concentration (mg/120 × 10^6 sperm)	Total motile sperm (%)	Progressively motile sperm (%)	PMI (%)
Control	0	33[c]	25[b]	16[c]
Methyl-β-cyclodextrin	1	39[a,b]	29[a]	29[a,b]
	2	48[a]	36[a]	39[a]
2-Hydroxypropyl- β-cyclodextrin	1	35[b]	27[a]	22[b]
	2	50[a]	38[a]	31[a]
SEM	8	7	5	

[a,b,c]Means with different superscripts within a column differ, $P < 0.05$.

Table 5
Percentages of motile sperm for up to 3 h of incubation at 38.5 °C after cryopreservation, when ram sperm were treated with 0, or 2 mg of either 2-hydroxypropyl-β-cyclodextrin (2HP) or methyl-β-cyclodextrin (MβC), each preloaded with cholesterol, prior to cryopreservation ($N = 7$).

Time (min)	Control sperm	MβC	2HP	SEM
0	35	37	48	8
30	48	45	63	9
60	37	37	56	8
90	33	36	52	8
120	34	31	53	8
180	26	29	45	7
SEM	8	9	7	

($P < 0.05$; Table 4), with the greatest difference observed in the 2-hydroxypropyl-β-cyclodextrin treated sperm.

3.4. Experiment 4. Resistance of sperm to incubation (sperm thermoresistance)

Sperm treated with 2-hydroxypropyl-β-cyclodextrin loaded with cholesterol prior to cryopreservation maintained greater percentages of total motile sperm (48%) than control (35%) or sperm treated with methyl-β-cyclodextrin loaded with cholesterol (37%; Table 5) immediately after thawing, and the CLC-treated samples maintained greater percentages of sperm motility for 3 h.

Table 6
Cholesterol content of sperm at different steps of the cryopreservation protocol (μg/10^6 sperm) when ram sperm were initially treated with 0, or 2 mg of either 2-hydroxypropyl-β-cyclodextrin (2HP) or methyl-β-cyclodextrin (MβC), each preloaded with cholesterol, prior to cryopreservation ($N = 4$).

Sperm treatment	Cholesterol content (μg/10^6 sperm)			
	Fresh sperm	Cooled sperm	Frozen-thawed sperm	SEM
Control	0.14^{a}	0.14^{a}	0.12^{a}	0.01
MβC	$0.38^{b,*}$	$0.22^{b,**}$	$0.22^{c,**}$	0.02
2HP	0.27^{b}	0.19^{b}	0.19^{b}	0.04
SEM	0.04	0.01	0.01	

^{a,b,c}Column means with different letters differ at $P < 0.05$.
*,**Row means with a different number of asterisks differ at $P < 0.05$.

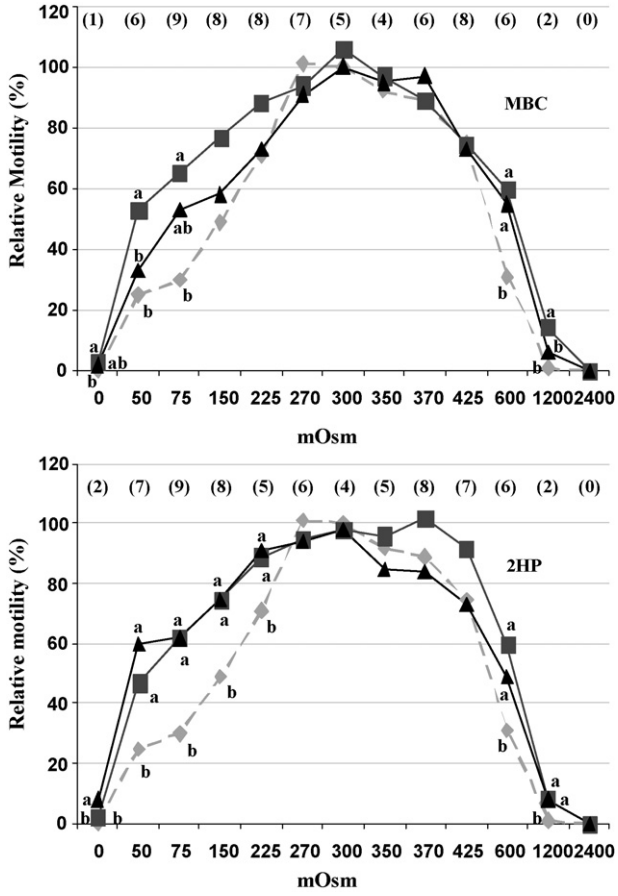

Fig. 2. Relative motility (percentage of motile sperm for each treatment in each osmotic diluent/percentage of motile sperm for control sample at 300 mOsm) of ram sperm treated with different concentrations of methyl-β-cyclodextrin (MβC) or 2-hydroxypropyl-β-cyclodextrin (2HP), each pre-loaded with cholesterol, placed into various anisosmotic solutions and then returned to isosmolality. –◆– Control sperm ■ 2 mg CLC/120 × 10^6 sperm ▲ 4 mg CLC/120 × 10^6 sperm. (a, b) Data in the same osmolality with different letters differ statistically ($P < 0.05$). The pooled standard error of the mean (SEM) for each osmotic diluent appears between brackets.

3.5. Experiment 5. Cholesterol content of CLC-treated ram sperm

The amount of cholesterol in ram sperm membranes was greater ($P < 0.05$) initially, after cooling, and after cryopreservation for sperm treated with either methyl-β- or 2-hydroxy-β-cyclodextrin each preloaded with cholesterol than for untreated sperm (Table 6). In addition, sperm cholesterol content decreased after cooling and again after cryopreservation, although this decrease was significant only for methyl-β-cyclodextrin treated sperm ($P < 0.05$; Table 6).

3.6. Experiment 6. Effect of CLC treatment on the osmotic tolerance of ram sperm

The relative motility of ram sperm decreased as cells were exposed to increasingly anisosmotic diluents (both hyper- and hypotonic; Fig. 2). When incubated in anisosmotic diluents, the relative motility of control sperm were different from sperm held in isosmotic conditions for diluents greater than 370 mOsm and for diluents less than 270 mOsm ($P < 0.05$). While sperm treated with 4 mg methyl-β-cyclodextrin benefited sperm only when cells were incubated in a diluent of 600 mOsm, treating sperm with 2 mg methyl-β-cyclodextrin or either 2 or 4 mg of 2-hydroxypropyl-β-cyclodextrin increased the osmotic tolerance of the ram sperm considerably ($P < 0.05$; Fig. 2)

3.7. Experiment 7. Zona binding assay

Samples treated with cholesterol-loaded 2-hydroxypropyl-β-cyclodextrin exhibited greater numbers of sperm binding to the zona pellucida than other treatments ($P < 0.05$; Table 7). In addition, sperm bound to oocytes of cattle and sheep equally.

4. Discussion

Although acceptable fertility rates using frozen-thawed ram sperm can be achieved, at the present time, they can only be achieved when coupled with laparoscopic intrauterine insemination (Salamon and Maxwell, 2000). This technique, however, is expensive and requires extensive training, which limits the practical use of cryopreserved ram sperm. New insemination protocols, or protocols that more efficiently cryopreserve ram sperm need to be developed for cryopreserved ram sperm to be used routinely. Current cryopreservation protocols for ram sperm result in roughly 40–60% motile sperm after thawing, but because of the latent damage incurred during cryopreservation, even fewer sperm are physiologically capable of fertilizing an oocyte (Salamon and Maxwell, 2000).

Table 7
Number of ram sperm bound to zona pellucida of bovine ($N = 14$) and ovine ($N = 9$) oocytes.

Sperm treatment	Bovine zona pellucida	Ovine zona pellucida	SEM
Control	4.6	9.3	2
Methyl-β-cyclodextrin	5.1	14.0	2
2-Hydroxypropyl-β-cyclodextrin	12.0*	27.3*	5
SEM	2	4	

N: number of drops (with five zona pellucida each) examined per sperm treatment. Seven ejaculates were analysed/sperm treatment. For the ovine zona pellucida, four ejaculates were analysed with two droplets with five zona each, and one ejaculate was analysed with just one droplet (five zona pellucida). Ovine zona pellucida were not used in two of the seven ejaculates.

* Indicates column mean is different from control at $P < 0.05$.

The cholesterol to phospholipid ratio of sperm membranes plays an important role in the resistance of the sperm to cold shock damage (Watson, 1981) and the cholesterol content of cell membranes can be modified using cyclodextrins (Christian et al., 1997; Combes et al., 2000; Purdy and Graham, 2004; Moore et al., 2005). When ram sperm were incubated with cyclodextrins that had not been pre-loaded with cholesterol, prior to cryopreservation, the percentages of motile sperm after thawing was less (31%) than control samples (43%), although the percentages of PMI cells were similar. The loss of motile cells, in these samples, was likely due to the cyclodextrin removing cholesterol from the sperm membranes prior to cooling, thereby making the sperm more sensitive to cooling damage than control cells. This would be consistent with the fact that sperm from species which have low cholesterol to phospholipid molar ratios are more sensitive to "cold shock" damage than sperm with higher ratios (Watson, 1981; Parks and Lynch, 1992). In addition, cholesterol efflux from sperm membranes contributes to signalling mechanisms that control sperm capacitation (Travis and Kopf, 2002), and the longevity of sperm in this pre-capacitated state is reduced.

The β-cyclodextrins exhibit the greatest affinity for removing cholesterol from human erythrocytes, followed by the α- and γ-cyclodextrins (Ohtani et al., 1989). However, we did not observe any differences in the cryosurvival of ram sperm treated with the α- or β-cyclodextrin, or the α- or β-cyclodextrin hydrate. Unmodified cyclodextrins such as α- or β-cyclodextrin have limited solubility in aqueous solutions (Pitha et al., 1988) which may explain the lack of treatment effect these CLCs had on sperm quality after cryopreservation, as they may not have been able to efficiently solubilize and transfer cholesterol into the cells. The ability of cyclodextrins to transfer cholesterol into cells is improved when their derivates are used (Pitha et al., 1988). Modifying cyclodextrins with methyl or hydroxypropyl residues enhance the solubility of the cyclodextrin in water and therefore its ability to solubilize hydrophobic compounds (Yancey et al., 1996), and several cyclodextrin derivates (hydrophilic, hydrophobic and ionic) have been developed to enhance the physico-chemical properties and inclusion capacity of natural cyclodextrins (Uekama, 2004). Methyl-β-cyclodextrin exhibited the greatest efficiency for accepting cholesterol from mouse L-cell fibroblasts, followed by 2-hydroxypropyl-β-cyclodextrin, while β-cyclodextrin exhibited the least cholesterol transfer efficiency (Yancey et al., 1996). When ram sperm were treated with either methyl-β- or 2-hydroxypropyl-β-cyclodextrin (each pre-loaded with cholesterol), sperm cryosurvival was improved compared to control sperm. In addition, while,

some authors reported methyl-β-cyclodextrin to be more efficient in adding cholesterol to various types of cells (Yancey et al., 1996; Christian et al., 1997), in the present study two CLCs benefitted ram sperm cryosurvival similarly.

The concentration of cholesterol-loaded methyl-β-cyclodextrin that optimized ram sperm cryosurvival was between 1 and 2 mg of CLC/120 × 10^6 sperm, which corresponds to 0.0083–0.017 mg CLCs/10^6 sperm. This amount is somewhat less, but similar to that reported for bull sperm (0.0125–0.025 mg CLCs/10^6 sperm; Purdy and Graham, 2004), and stallion sperm (0.0125–0.026 mg CLCs/10^6 sperm; Combes et al., 2000; Moore et al., 2005) using this cyclodextrin. When sperm were treated with 1 or 2 mg/120 × 10^6 sperm of either methyl-β-cyclodextrin or 2-hydroxypropyl-β-cyclodextrin (each pre-loaded with cholesterol), sperm cryosurvival improved with respect to control sperm (sperm that had not been treated with CLC). The increases observed in both the percentage of motile sperm (15–17%) after treatment with 2 mg of these CLCs were similar to those reported for bull sperm treated with 1.5 to 3 mg methyl-β-cyclodextrin/120 × 10^6 sperm (+15–18% total motile sperm and 9–14% PMI sperm; Purdy and Graham, 2004), stallion sperm treated with 1.33 mg methyl-β-cyclodextrin/50 × 10^6 sperm (+15–19% total motile sperm and +17–21% PMI sperm; Combes et al., 2000), and stallion sperm treated with 1.5 mg methyl-β-cyclodextrin/120 × 10^6 sperm (+10% total motile sperm and +9% PMI sperm; Moore et al., 2005). However, greater sperm cryosurvival rates were observed for sperm treated with 2 mg 2-hydroxypropyl-β-cyclodextrin compared to 1 mg of this CLC.

Treating raw ejaculates with 2 mg/120 × 10^6 sperm of either methyl-β-cyclodextrin or 2-hydroxypropyl-β-cyclodextrin (pre-loaded with cholesterol), resulted in greater percentages of total motile sperm after cryopreservation, than untreated sperm. However, only for sperm treated with 2-hydroxypropyl-β-cyclodextrin were the percentages of PMI cellsgreater. The reason for this phenomenon is unknown, especially because the previous experiments showed increased sperm cryosurvival after ram sperm were treated with cholesterol-loaded methyl-β-cyclodextrin. Perhaps at the greater sperm concentrations that occur in undiluted ejaculates the methyl-β-cyclodextrin is not as effective as the 2-hydroxypropyl-β-cyclodextrin in transferring cholesterol, and greater CLC concentrations, longer incubation times or increased temperature of incubation is needed to achieve optimal cholesterol transfer into ram sperm. This improvement is particularly interesting because the cryosurvival rates in control samples of the present study are similar to others who used similar sperm dilution rates (Kumar et al., 2003; Purdy, 2006), although less than for procedures using lesser sperm dilution rates (Awad and Graham, 2004; Martínez-Pastor et al., 2004; Marco-Jiménez et al., 2005). The reason for this discrepancy is likely due to the dilution rate used. Salamon and Maxwell (2000) reported that ram sperm quality after cryopreservation is greatly affected by the initial dilution rate of the semen. The initial dilution rate, prior to freezing, in the present study and first studies mentioned, ranged from 11- to 26-fold, while those used by Awad and Graham (2004), Martínez-Pastor et al. (2004) and Marco-Jiménez et al. (2005) were only 2- to 5-fold (v/v). It is possible that seminal plasma proteins help prevent cold-shock damage to ram sperm (Pérez-Pé et al., 2001), and that sperm diluted as we described would be exposed to less of these proteins.

The discrepancy between the percentage of plasma membrane intact sperm and total motile sperm observed in most of these experiments (approximately 15% fewer plasma membrane intact sperm than motile sperm) is likely due to the dilution process sperm underwent during the sperm viability evaluation. While sperm motility was evaluated in non-diluted sperm, the sperm viability was evaluated after 4.5 fold dilution, a process which induces osmotic stress to cell membranes and likely damaged some of the initially viable, but membrane compromised, sperm.

Cells are exposed to many stresses during cryopreservation which can cause minor to severe damage (Mazur, 1984; Amann and Pickett, 1987; Hammerstedt et al., 1990; Amann, 1999). Some of the stresses sperm are exposed to during the cryopreservation process are due to osmotic changes across the plasma membrane. For example, penetrating cryoprotectants, such a glycerol, are often required for adequate cell survival. However, the addition of these cryoprotectants to cells and the removal of them from the cells, induce anisosmotic environments that cause the cells to undergo potentially damaging volume changes (Hammerstedt et al., 1990; Ball and Vo, 2001; Guthrie et al., 2002). In addition, as water freezes during the cryopreservation process, solute concentrations in unfrozen water channels, where the cells reside, increases and the sperm make significant volume adjustments (Amann and Pickett, 1987; Hammerstedt et al., 1990; Amann, 1999). This process is reversed when the solution is thawed (Hammerstedt et al., 1990). After thawing, when sperm that are loaded with cryoprotectant (having an internal osmolality of approximately 1300 mOsm, if 1 M cryoprotectant was used) are transferred to an isosmotic environment of approximately 300 mOsm (*in vitro* medium or female reproductive tract fluid) a 2.3-fold overall increase in cell volume can result, before cryoprotectant can exit the cell and reach equilibrium (Hammerstedt et al., 1990).

One way to lessen cell damage incurred during cryopreservation is to widen the osmotic tolerance of the cells. We report that ram sperm have narrow osmotic tolerance limits, similar to those of boar and bull sperm (Guthrie et al., 2002) and rapidly become immotile when exposed to anisosmotic conditions less than 270 mOsm or greater than 370 mOsm. Adding cholesterol to ram sperm membranes widened the osmotic tolerance limit of the sperm both in the hypertonic range, but even more dramatically in hypotonic solutions. This increased tolerance to osmotic changes is likely responsible for at least some of the improvement seen in the cryosurvival rates of CLC-treated ram sperm.

Cholesterol has a profound effect on the thermodynamic and mechanical properties of the lipid bilayers, and influences stability and fluidity (Sparr et al., 2002). When temperature is reduced below the transition temperature of the membrane, the lipids within the membrane undergo a phase change from the liquid state to the gel state (rigid form). The temperature at which this phase

transition occurs depends upon the lipid species which compose the membrane (Amann, 1999), but because biological membranes are composed of many different lipids, the membrane undergoes this transition over a temperature range, with some lipids transitioning to the gel state at the greatest temperature of the range, and by the time the least temperature of the range is reached, all the lipids are in the gel state (Moran et al., 1992). At a temperature within the phase transition range of a membrane, when some of the lipids are in the gel state and some in the fluid state, a membrane phase separation is observed, which can result in the loss of intracellular molecules, which for sperm can result in irreversible motility loss (Drobnis et al., 1993).

Cholesterol modulates the fluidity of membranes by interacting with the fatty acyl chains of the phospholipids and the cholesterol:phospholipid ratio is an important determinant of membrane fluidity (Watson, 1981). This ratio is very high (0.88–0.99) in sperm from species whose sperm are resistant to cold shock (rabbit and human), and low (0.38 and 0.45) in sperm from species whose sperm are susceptible to cold shock (bull and ram; Darin-Bennett and White, 1977). Treating ram sperm with CLC increased the cholesterol content in fresh sperm between 1.93- and 2.7-fold, similar to the increase reported for bull (Purdy and Graham, 2004) and stallion (Moore et al., 2005) sperm treated with methyl-β-cyclodextrin preloaded with cholesterol.

The reason that 2-hydroxypropyl-β-cyclodextrin transferred cholesterol more efficiently into the ram is not known, although this may be due to the differences in the molecule side chains with the hydroxypropyl group (versus a methyl group) allowing the molecule to interact with the glycocalyx of the ram sperm more effectively permitting more efficient transfer. This may be due to specific ram sperm membrane proteins or carbohydrates as bull sperm cryosurvival rates were more benefitted when treated with methyl-β-cyclodextrin than with 2-hydroxypropyl-β-cyclodextrin (Graham unpublished). In contrast, Yancey et al. (1996) reported that methyl-β-cyclodextrin more efficiently removed cholesterol from mouse L-cell fibroblasts than 2-hydroxypropyl-β-cyclodextrin or β–cyclodextrin.

Untreated ram sperm lost 14% of their cholesterol after cooling, freezing and thawing. Other authors have observed changes in the lipid composition of sperm membranes after cryopreservation (Buhr et al., 1994; Cerolini et al., 2001; Maldjian et al., 2005), including a loss of cholesterol (Cerolini et al., 2001; Maldjian et al., 2005; Moore et al., 2005). This lesser cholesterol content in cryopreserved sperm may have an impact on cryopreserved sperm longevity, since one of the first steps in capacitation is the removal of cholesterol from the membrane (Cross, 1998; Visconti and Kopf, 1998). After cholesterol is removed from the membrane, membrane fluidity increases and membrane proteins can undergo rearrangements within the membrane that lead to sperm capacitation and the acrosome reaction (Flesch et al., 2001; Travis and Kopf, 2002). Cryopreservation induces similar capacitation-like changes in sperm (Bailey et al., 2000), part of which are likely due to this loss of membrane cholesterol.

Sperm cryoinjury, including the capacitation-like changes induced in frozen-thawed sperm, lead to shortened sperm longevity and impaired sperm transport in the female genital tract (Bailey et al., 2000). Therefore, cryopreserved sperm are generally inseminated in the upper female genital tract (intrauterine or intraoviductal) and near the time of ovulation. Uuntreated ram sperm exhibited a decline of about 50% in motility over the 3 h incubation, similar to results reported by Aisen et al. (2002), while sperm treated with 2-hydroxypropyl-β-cyclodextrin prior to cryopreservation maintained about 66% of their motility at 20 min over the 3 h incubation period. Therefore, maintaining greater cholesterol concentrations in the sperm, may decrease the capacitation-like changes that occur when sperm are frozen, which in turn increases the longevity of the cells after thawing; something that may prove important in increasing the fertilizing capacity of cryopreserved sperm.

Because CLC-treated sperm contain more cholesterol after thawing than untreated sperm and since the loss of membrane cholesterol is part of capacitation, it is possible that capacitation may take longer in CLC-treated sperm. An *in vitro* assay to evaluate the capacitation status of CLC-treated sperm revealed that added cholesterol did not hinder the sperm from binding to zona pellucidae of either sheep or cattle oocytes. Obtaining large numbers of homologous oocytes is difficult for many species, consequently heterologous oocytes are sometimes used (Sinowatz et al., 2003) and similar numbers of ram sperm bound to the sheep oocytes and cattle oocytes. In addition, Slavik et al. (1990) reported that ram sperm could penetrate oocytes of cattle, although sperm penetration rates were less for ram sperm (67%) than for bull sperm (83%). Therefore, oocytes of cattle can be used to evaluate ram sperm quality *in vitro*.

The exact mechanism by which cholesterol improves sperm cryosurvival is still not known. The additional cholesterol may broaden the phase transition of the sperm membranes, thereby reducing lipid of the same species from aggregating into specific domains within the membrane (Drobnis et al., 1993), as well as increasing membrane fluidity at lower temperatures (Purdy et al., 2005). By increasing the cholesterol:phospholipid ratio of ram sperm, sperm membranes may exhibit reduced membrane phase separations and therefore reduced leakage of cellular components (such as potassium) from the cell (Drobnis et al., 1993) or it may inhibit calcium entry into the sperm, which is a prerequisite for capacitation and/or senescence (Visconti et al., 1999). However, by understanding the mechanism by which cholesterol addition affects sperm membranes a greater understanding of membrane physiology may be gained during cryopreservation, and develop even better techniques for cryopreserving sperm.

In conclusion, treating ram sperm with CLC sperm prior to cryopreservation benefits sperm cryosurvival similarly to other species with sperm that have lesser cholesterol:phospholipid ratios, such as horses (Combes et al., 2000; Moore et al., 2005), cattle (Purdy and Graham, 2004; Mocé and Graham, 2006) and donkeys (Alvarez et al., 2006). In addition, the CLCs can be added to the raw ejaculates, making this new technology practical for application in the industry, using current cryopreservation techniques. These sperm exhibit greater cholesterol concentrations prior to and after cooling, freezing and thawing, they exhibit wider

osmotic tolerance limits than untreated sperm, which may play a role in more sperm surviving cryopreservation. CLC-treated sperm remain motile longer than untreated sperm after thawing and show increased capacity to bind to the zona pellucida. However, no *in vitro* assay accurately estimates the *in vivo*-fertilizing potential of sperm (Amann, 1989, 2005; Amann and Hammerstedt, 1993; Foote, 2003; Graham and Mocé, 2005), therefore, the *in vivo* fertility of CLC-treated sperm needs to be determined.

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